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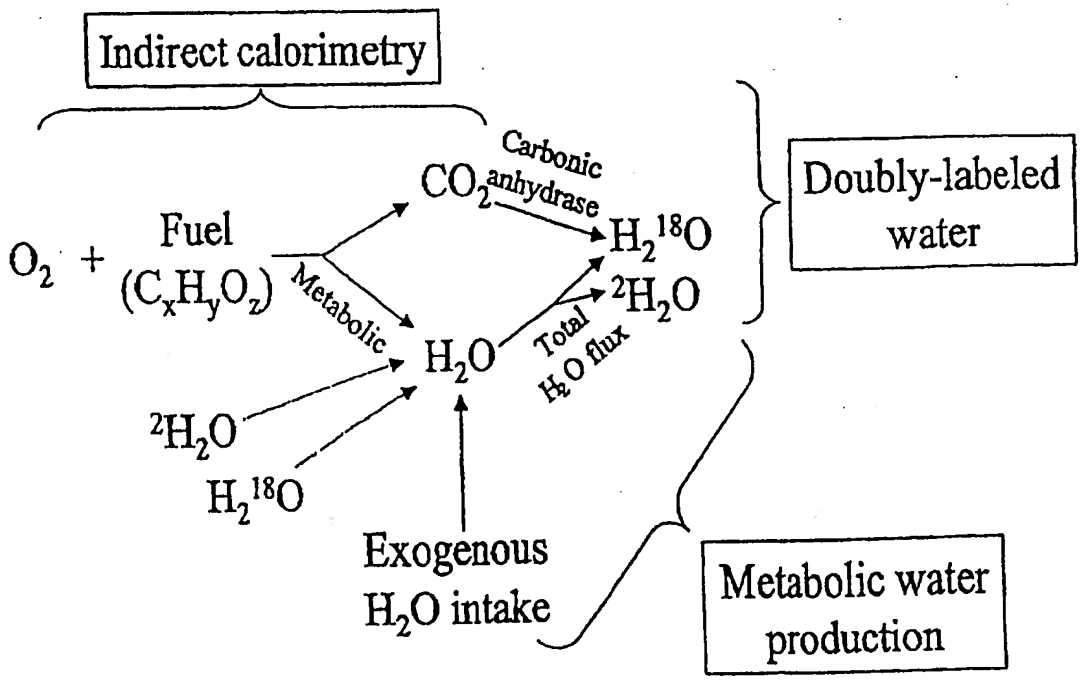
(54) Title: METHODS AND KITS FOR DETERMINING ENERGY EXPENDITURE IN LIVING ORGANISMS BY METABOLIC WATER PRODUCTION



(57) Abstract: The invention relates to the measurement of energy expenditure. More specifically, it relates to methods of using labeled water to measure metabolic water production and thereby measure energy expenditure in an individual or one or more cells, such as a cell culture.

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METHODS AND KITS FOR DETERMINING ENERGY EXPENDITURE IN LIVING ORGANISMS BY METABOLIC WATER PRODUCTION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. patent application 60/392,072 filed on June 26, 2002 which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0001] The invention relates to the measurement of energy expenditure. More specifically, it relates to methods of using labeled water to measure energy expenditure in an individual from the rate of metabolic water production.

BACKGROUND OF THE INVENTION

[0002] Measurement of energy expenditure has been accomplished by a variety of methods. The most widely used current techniques involve complex, expensive instrumentation (*e.g.*, indirect or direct calorimeters) that cannot be used easily over the long-term, are impractical for large numbers of animals or people, and are not easily applied in small animals. Previous attempts to measure energy expenditure has focused on measuring carbon dioxide production and/or oxygen consumption. See, for example, Schoeller, *J. Nutrition* 118: 1278-1289 (1988); Ferrannini *Metabolism* 37:287-301 (1988); and Jequier et al. *Ann. Rev. Nutr.* 7:187-208 (1987). The difficulties of measuring energy expenditure by monitoring carbon dioxide production is documented in these and other publications.

[0003] Research on thermogenic agents for the treatment of obesity and on genes or hormones that affect energy expenditure, would be particularly benefited by a simple measurement technique for total energy expenditure that could be applied to very large numbers (*e.g.*, thousands) of animals or people.

[0004] The ability to measure total energy expenditure easily and inexpensively in living organisms would be of great value for research as well as medical care. The invention described herein provides these advantages.

BRIEF SUMMARY OF THE INVENTION

[0005] To meet these needs, the invention described herein provides methods of determining energy expenditure by measuring metabolic water production. Accordingly, in one aspect, the invention provides a method of determining energy expenditure in an individual by (a) administering to the individual an amount of labeled exogenous water for a time sufficient for the label to reach a steady-state isotopic content in the individual; (b) obtaining a biological sample from the individual; (c) measuring isotopic content of water in the biological sample; (d) comparing the isotopic content of water in the biological sample to the isotopic content in the labeled exogenous water administered to the individual in step (a) to determine the dilution rate of the label in total body water; and (e) calculating the production rate of metabolic water from the dilution rate to determine the energy expenditure in the individual.

[0006] In another aspect, the invention presents a method of determining energy expenditure in an individual by (a) administering labeled water to the individual; (b) discontinuing the administering step (a); (c) obtaining a biological sample from the individual; (d) measuring isotopic content of water in the biological sample; (e) determining the decay rate of labeled water to determine the total dilution rate of body water in the individual; and (f) calculating the production rate of metabolic water to determine the energy expenditure in the individual.

[0007] The invention also provide methods of determining energy expenditure of one or more cells *in vitro* by (a) incubating the one or more cells *in vitro* in a medium containing a known isotopic content of labeled water; (b) obtaining a sample of cellular medium after a known period of time; (c) measuring the isotopic content of water in the sample of cellular medium; (d) calculating the rate of decline in the isotopic content of the labeled water in the cellular medium over the known period of time; and (e) calculating the production rate of metabolic water by one or more cells to determine the energy expenditure in the one or more cells.

[0008] In other aspects of the methods, the labeled water may be $^{2}H_2O$, $^{3}H_2O$, or H_2O^{18}. The labeled water may be administered by methods known in the art, including orally. In a further aspect, the biological sample may be any known bodily sample, including urine, blood, saliva, interstitial fluid, edema fluid, lacrimal fluid, inflammatory exudates, synovial fluid, abscess, emphysema, cerebrospinal fluid, sweat, pulmonary secretions, seminal fluid, feces, bile, and intestinal secretions. In an additional aspect, the isotopic enrichment of in the labeled water may be determined by analytic techniques including but not limited to isotope-ratio mass spectrometry, gas chromatography/mass spectrometry (GC/MS), cycloidal/MS, or Fourier-Transform Infrared Spectroscopy (FTIR). In yet another aspect, the individual may be a rat, mouse, other experimental animal or human.

[0009] In another aspect, the invention provides a method for identifying a pharmacologic agent having a thermogenic action by (a) determining the energy expenditure in an individual prior to any exposure to the pharmacologic agent; (b) exposing the individual to the pharmacologic agent; (c) determining the energy expenditure in the individual after exposure to the pharmacologic agent; and (d) comparing the energy expenditure in the individual before and after exposure to the agent, wherein an increase in energy expenditure after exposure to the pharmacologic agent identifies the pharmacologic agent as having a thermogenic action.

[0010] In another aspect, the invention provides a method for identifying a pharmacologic agent having thermogenic action by (a) determining the energy expenditure in one or more cells prior to exposure to the pharmacologic agent; (b) exposing the one or more cells to the pharmacologic agent; (c) determining the energy expenditure in the one or more cells after exposure to the pharmacologic agent; and (d) comparing the energy expenditure in said one or more cells before and after exposure to the agent, wherein an increase in energy expenditure after exposure to the pharmacologic agent identifies the pharmacologic agent as having a thermogenic action.

[0011] In another aspect, the invention provides a method for identifying one or more genes involved in a thermogenic action in an individual experimental animal or human by (a) determining the energy expenditure in the individual, wherein the individual has been genetically manipulated or an individual or is genetically well-characterized; and (b) correlating the energy expenditure in the individual with the genetic composition or gene expression of the individual to thereby identify one or more genes involved in a thermogenic action in the individual.

[0012] In another aspect, the invention provides a method for identifying one or more genes that are involved in thermogenic actions in one or more cells by (a) determining the energy expenditure in the one or more cells, wherein the one or more cells are selected from the group consisting of a cell type, one or more cells that have been genetically manipulated, or one or more cells that are genetically well-characterized; and (b) correlating the energy expenditure in the one or more cells with the genetic composition or gene expression of the one or more cells to thereby identify one or more genes involved in thermogenic actions in the one or more cells.

[0013] In another aspect, the invention provides a method for identifying the presence of negative caloric balance in an individual by (a) determining metabolic water production and labeled-water enrichment of body water in an individual prior to exposure to an intervention; (b) subjecting the individual to an intervention; (c) measuring metabolic water production and ^2H_2O enrichment of body water after the intervention; and (d) monitoring ^2H_2O enrichment of body water relative to drinking water, wherein a decline in ^2H_2O enrichment of body water relative to drinking water is indicative of fat mobilization and identifies a negative whole-body caloric balance. In a further aspect, the intervention may be administration of an agent, the presence of one or more transgenes, or participation in an exercise regimen or dietary regimen.

[0014] In another aspect, the invention provides a method of identifying or monitoring a disease or disorder by a) determining the energy expenditure of the individual at a first timepoint; and b) determining the energy expenditure of an

individual at a second timepoint; wherein a change the energy expenditure between the first timepoint and the second timepoint identifies the disease or disorder. In a further aspect, the disease or disorder may be diabetes mellitus or obesity or other disorder related to energy balance.

[0015] In another aspect, the invention provides methods of identifying a beneficial effect of an exercise regimen by a) determining the energy expenditure of the individual at a first timepoint; b) subjecting the individual to an exercise regimen; and c) determining the energy expenditure of an individual at a second timepoint; wherein a change in the energy expenditure between the first timepoint and the second timepoint identifies a beneficial effect of the exercise regimen.

[0016] In another aspect of the invention, the invention also includes a kit for determining energy expenditure in an individual or cells including labeled water and instructions for using the kit. The kit may further include instructions for performing energy expenditure calculations, tools for administering administering labeled water, tools for obtaining biological samples.

BRIEF DESCRIPTION OF THE FIGURES

[0017] Figure 1 is a schematic of metabolic water production method compared to other methods for measuring energy expenditure.

[0018] Figure 2 shows the results of an animal study wherein rats were administered ^2H_2O over a period of weeks. The Y axis is the percent of ^2H_2O enrichment, and the X-axis is the number of weeks.

[0019] Figure 3 shows the effects of recombinant leptin administration. Figure 3(A) depicts the effect of recombinant leptin administration on metabolic water production. The Y-axis represents the rate of molecular water production (MWP) in mL per day, and the X-axis represents the days of leptin treatment. 3(B) depicts ^2H_2O enrichments in urine (average values) and 3(C) ^2H_2O enrichments in urine (individual animals). In 3(B) and 3(C), the Y-axis represents ^2H_2O Enrichment in molar percent excess (MPE) and the X-axis depicts the days of leptin treatment at each measurement.

[0020] Figure 4 shows body ^2H_2O enrichments in sample subjects. Subjects 1 and 2 (left side) are healthy individuals. Subjects 3 and 4 (right side) are individuals with HIV/AIDS.

[0021] Figure 5 shows body ^2H_2O enrichment in individuals administered with ^2H_2O drinking water.

DETAILED DESCRIPTION OF THE INVENTION

[0022] The invention provides, *inter alia*, methods for determining total energy expenditure by using labeled water.

I. General Techniques

[0023] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook et al., 1989) Cold Spring Harbor Press; *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984*); Methods in Molecular Biology, Humana Press; Cell Biology: A Laboratory Notebook* (J.E. Cellis, ed., 1998) Academic Press; *Animal Cell Culture* (R.I. Freshney, ed., 1987); *Introduction to Cell and Tissue Culture* (J.P. Mather and P.E. Roberts, 1998) Plenum Press; *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., 1993-8) J. Wiley and Sons; *Methods in Enzymology* (Academic Press, Inc.); *Handbook of Experimental Immunology* (D.M. Weir and C.C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller and M.P. Calos, eds., 1987); *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); *Current Protocols in Immunology* (J.E. Coligan et al., eds., 1991); *Short Protocols in Molecular Biology* (Wiley and Sons, 1999); and *Mass isotopomer distribution analysis at eight years: theoretical, analytic and experimental considerations* (Hellerstein and Neese, *Am J Physiol* 276 (*Endocrinol Metab.* 39) E1146-E1162, 1999).

II. Definitions

[0024] Unless otherwise defined, all terms of art, notations and other scientific terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art. The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, *Mass isotopomer distribution analysis at eight years: theoretical, analytic and experimental considerations* by Hellerstein and Neese (*Am J Physiol* 276 (*Endocrinol Metab.* 39) E1146-E1162, 1999). As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in accordance with manufacturer defined protocols and/or parameters unless otherwise noted.

[0025] "Total energy expenditure" and "energy expenditure" are used interchangeably and refer to the fuel oxidation rate or caloric consumption of an individual or cells.

[0026] "Fuel oxidation rate" as used herein refers to the rate at which fuel is oxidized by the cell ultimately using molecular oxygen and producing catabolic products (including, but not limited to, water and carbon dioxide).

[0027] "Caloric consumption" as used herein is used synonymously with "fuel oxidation rate", to represent the same processes.

[0028] "Metabolic water" refers to water (H_2O) produced by a cell or individual during oxidative metabolism. Molecular oxygen reacts with reduced hydrogen derived from fuels to produce metabolic water.

[0029] "Exogenous water" refers to water acquired from outside a cell or individual, for example, by drinking by an individual. Exogenous water differs from water produced during oxidative metabolism in an individual or cell.

[0030] "Total body water" refers to the total pool of water, derived from combined inputs of metabolic water and exogenous water present in an individual.

[0031] "Labeled Water" includes water labeled with one or more specific heavy isotopes of either hydrogen or oxygen. Specific examples of labeled water include $^{2}H_2O$, $^{3}H_2O$, and $H_2{}^{18}O$.

[0032] "Total dilution rate" refers to the dilution rate of labeled water in an individual that occurs due to both intake of exogenous water and production of metabolic water.

[0033] "Isotopes" refer to atoms with the same number of protons and hence of the same element but with different numbers of neutrons (*e.g.*, H vs. or ^{2}H or D).

[0034] An "individual" is a animal, preferably a vertebrate, more preferably a mammal, more preferably a human. Mammals include, but are not limited to, farm animals, sport animals, pets, primates, mice and rats.

[0035] A "biological sample" encompasses a variety of sample types obtained from an individual. The definition includes, but is not limited to, blood, urine, saliva, lacrimal fluid, and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom, and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components. The term "biological sample" encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples.

[0036] "Biological fluid" refers to any biological fluid known in the art. The term "biological fluid" includes, but is not limited to urine, blood, interstitial fluid, edema fluid, saliva, lacrimal fluid, inflammatory exudates, synovial fluid, abscess, emphysema or other infected fluid, cerebrospinal fluid, sweat, pulmonary secretions (sputum), seminal fluid, feces, bile, intestinal secretions, or other bodily fluid.

III. Methods of the Invention

[0037] Energy production in mammals is generated by reactions involving fuel and, ultimately, molecular oxygen. A consequence of energy producing reactions is the production of catabolic products, including water, carbon dioxide, and other byproducts (*e.g.*, nitrogenous end-products like urea, partial oxidation productions like ketone bodies, etc.). The amount of energy expenditure is directly related to the fuel oxidation rate (caloric consumption rate) in an individual. Previous methods of measuring energy expenditure have focused either on carbon dioxide production and oxygen consumption (*i.e.*, gas exchange) or on heat production (*i.e.*, direct calorimetry). The invention disclosed herein provides methods of measuring energy expenditure in a reliable, reproducible, high-throughput and non-invasive manner by measuring the water production component of the energy expenditure reaction. The production of water from energy-yielding biochemical pathways generally parallels the production of carbon dioxide (figure 1) and, by analogy, can be used as a measure of energy expenditure. The production of this "metabolic water," as distinguished from exogenous water taken in by an individual by mouth, is measured by the invention described herein.

[0038] Two isotopic labeling methods can be used to measure total energy expenditure or fuel oxidation rate in an individual, based on metabolic water production. The two general methods of labeling that may be used are steady-state or die-away curve.

[0039] The general premise behind steady-state labeling is that the input of label into a system is kept at a steady-state during the time of the measurements. For measuring energy consumption of an individual using steady-state labeling kinetics, the following steps are followed: (1) administer labeled water to the individual repeatedly or continuously for a period of time sufficient to reach a steady-state level of isotopic content of body water in the individual; (2) obtain a biological sample containing body water from the individual; (3) measure the isotopic content of hydrogen or oxygen (*i.e.*, enrichment of ^{2}H or ^{18}O or specific activity of ^{3}H) of the water in the biological sample; (4) compare the isotopic content of hydrogen or

oxygen of the water in the biological sample to the isotopic content of the administered hydrogen- or oxygen-labeled water, to calculate the total dilution rate of the label due to the combined inputs of both ingested (exogenous) water plus metabolic (endogenous) water; (5) calculate the production rate of metabolic water based on the total dilution by metabolic and exogenous water, corrected for the daily intake or input of exogenous water; and (6) convert the production rate of metabolic water to the total energy expenditure, or whole-body fuel oxidation rate, in the individual, using equations derived and expressed in tabular form herein.

[0040] For measuring the total energy expenditure of an individual using the label die-away method, the following steps are followed: (1) administer labeled water to the individual without restriction as to timing, pattern or duration (*e.g.*, as a single pulse dose, repeated doses, or continuously); (2) discontinue administration of labeled water to the individual; (3) obtain two or more biological samples containing body water from the individual at known times after discontinuing administration of labeled water; (4) measure the isotopic content of hydrogen or oxygen (*e.g.*, enrichment of ^{2}H or ^{18}O or specific activity of ^{3}H) of the water in the biological samples; (5) calculate the decay rate of labeled water in the individual to establish total dilution rate of body water in the individual due to combined inputs of both ingested (exogenous) water plus metabolic (endogenous) water; (6) calculate the production rate of metabolic (endogenous) water from the total dilution rate of body water, corrected for the daily intake or input of exogenous water; and (7) convert the production rate of metabolic water to total energy expenditure (whole-body fuel oxidation rate), using tables that Applicants have derived and expressed in tabular form herein.

[0041] The measurement of energy expenditure is also applicable to smaller systems than an individual, such as one or more cells (e.g. cultured cells). In one embodiment, the total energy expenditure of one or more cells *in vitro* can be determined by the following steps: (1) incubate the cells *in vitro* in a medium containing a known isotopic content of labeled water in a closed system (*i.e.*, a system not open to exchange with external water, such as a cell culture); (2) obtain a sample of or cellular medium after a known period of time; (3) measure the isotopic

content of the labeled water (*i.e.*, enrichment of ^{2}H or ^{18}O or specific activity of ^{3}H) in the sample of cellular medium; (4) calculate the rate of decline in the isotopic content of the labeled water over the measured time interval in the medium, to determine the production rate of endogenous water (metabolic water) by the cells; and (5) convert the production rate of metabolic water to a total energy expenditure (fuel oxidation rate), using equations that Applicants have derived and expressed in tabular form. The invention is not limiting to the type of cell that can be used. Any type of cell maybe used, including, but not limited to, a cell line that has been propagated for many generations, primary cells obtained from tissue samples, cancerous cells, non-cancerous cells, fetal cells, or adults cells. The one or more cells may be in cell culture. It is understood that the medium used for culturing the cells is suitable to promote survival and growth of such cells.

Administering Labeled Water

[0042] For the methods described above (*e.g.*, steady-state method or die away method), water can be labeled on the hydrogen (*e.g.*, $^{2}H_2O$ or $^{3}H_2O$) or on the oxygen (*e.g.*, $H_2{}^{18}O$). Methods of labeling water are well-known in the art. Several commercial sources of $^{2}H_2O$ or $H_2{}^{18}O$ are available, including Isotec, Inc. (Miamisburg OH, catalogue #82-709-01-5), Deuterium oxide (100%) and Cambridge Isotopes, Inc. (Andover MA, catalogue #DLM-2259-70-1), and Deuterium oxide (70%). The labeled water is administered at a known isotopic content (*e.g.*, by enrichment or specific activity) to an individual and the volume of water administered is monitored. The labeled water can be administered by various methods including, but not limited to, orally (*e.g.*, drinking), parenterally (e.g. intravenous infusions), subcutaneously, intravenously, and intraperitoneally. In one embodiment, the labeled water is $^{2}H_20$ that is administered by drinking. The isotopic content of labeled water that is administered can range from about 0.001% to about 20% and depends upon the analytic sensitivity of the instrument used to measure isotopic content of the labeled water. In another embodiment, the individual is a rat and a specific enrichment of $^{2}H_2O$ (e.g. 4% $^{2}H_2O$) is administered as drinking water. In another embodiment, the individual is a human and a specific daily dose (e.g. 50 ml) of $^{2}H_2O$ is administered. The labeled water should be administered at a level at

which detection on a suitable instrument can be measured. For steady-state methods, the labeled water is optimally administered for a period of sufficient duration to achieve steady-state plateau (*e.g.*, 3-8 weeks in humans, 1-2 weeks in rodents). For die-away methods, the labeled water is administered for a period of sufficient duration to achieve detection of a change in the label. This can be determined empirically by sampling of biological fluids at intervals.

Obtaining One or More Bodily Samples

[0043] For both continuous labeling and discontinuous labeling methods, a biological sample is obtained from bodily fluids or tissues of an individual. Specific methods of obtaining biological samples are well known in the art. Bodily fluids include, but are not limited to, urine, blood, saliva, interstitial fluid, edema fluid, lacrimal fluid, inflammatory exudates, synovial fluid, abscess, emphysema, cerebrospinal fluid, sweat, pulmonary secretions, seminal fluid, feces, bile, and intestinal secretions. The fluids may be isolated by standard medical procedures known in the art. The isotopic content of water in these biological samples is determined and compared to the isotopic content of the initial labeled water prior to administration.

[0044] The frequency of biological sampling can vary depending on different factors. Such factors include, but are not limited to, ease of sampling, nature of an intervention; or half-life of a drug used in a treatment if monitoring responses to treatment.

Determining the Isotopic Content of Labeled Water

[0045] For both continuous and discontinuous labeling methods, labeled water may be measured by various methods such as mass spectrometry, particularly gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR), or liquid scintillation counting, and Fourier-Transform Infrared Spectroscopy (FTIR).

Mass Spectrometry

[0046] Mass spectrometers convert components of a sample into rapidly moving gaseous ions and separate them on the basis of their mass-to-charge ratios. The distributions of isotopes or isotopologues of ions, or ion fragments, may thus be used to measure the isotopic enrichment.

[0047] Generally, mass spectrometers comprise an ionization means and a mass analyzer. A number of different types of mass analyzers are known in the art. These include, but are not limited to, magnetic sector analyzers, electrostatic analyzers, quadrapoles, ion traps, time of flight mass analyzers, and fourier transform analyzers. In addition, two or more mass analyzers may be coupled (MS/MS) first to separate precursor ions, then to separate and measure gas phase fragment ions.

[0048] Mass spectrometers may also include a number of different ionization methods. These include, but are not limited to, gas phase ionization sources such as electron impact, chemical ionization, and field ionization, as well as desorption sources, such as field desorption, fast atom bombardment, matrix assisted laser desorption/ionization, and surface enhanced laser desorption/ionization.

[0049] In addition, mass spectrometers may be coupled to separation means such as gas chromatography (GC) and high performance liquid chromatography (HPLC). In gas-chromatography mass-spectrometry (GC/MS), capillary columns from a gas chromatograph are coupled directly to the mass spectrometer, optionally using a jet separator. In such an application, the gas chromatography (GC) column separates sample components from the sample gas mixture and the separated components are ionized and chemically analyzed in the mass spectrometer.

[0050] Isotopes from water may be converted to other molecules, which are then detected by mass spectrometry. For example, hydrogens on water may be converted to acetylene prior to detection by mass spectrometry. The acetylene may be derivatized to tetrabromethane.

Liquid Scintillation Counting

[0051] Radioactive isotopes may be observed using a liquid scintillation counter. Radioactive isotopes such as ^3H emit radiation that is detected by a liquid scintillation detector. The detector converts the radiation into an electrical signal, which is amplified. Accordingly, the number of radioactive isotopes in water may be measured.

Calculation of total dilution or turnover of body water

[0052] The comparison of isotopic content before and after administration to an individual allows the calculation of the total dilution of body water by use of the following equation:

$$\text{Total dilution of body } H_2O \text{ (ml/day)} = \frac{\text{Daily } H_2O \text{ intake (ml/day)}}{(\text{Enrichment body } H_2O/\text{Enrichment of drinking } H_2O)}$$

[0053] Total dilution refers to the dilution of labeled water that occurs due to both intake of exogenous water and production of metabolic water, the latter during energy expenditure as oxygen reacts with reduced hydrogens derived from fuels to produce water (Figure 1). The total dilution can then be used to calculate the production rate for metabolic water. This is done by subtracting exogenous water intake (drinking water) from total dilution of body water, calculated by the equation shown above. Alternatively, the production rate of metabolic water can be calculated directly, using the following equation:

$$\text{Production rate of metabolic water (ml/day)} = \frac{b\,(1-R)}{R}$$

where b = exogenous water intake (ml/day) and R = the ratio of labeled water enrichment in body water/labeled water enrichment in drinking water. Care should be taken to correct for daily intake of exogenous water (*e.g.*, moist foods, other non-labeled drinks).

Once the production rate for metabolic water is obtained, this rate can be converted to whole-body fuel oxidation rate, or total energy expenditure, by applying

the formula:

Total energy expenditure (kCal/day) = (Metabolic water production, ml/day)·(Caloric value/ml H_2O produced)

where Table 1 gives caloric value per ml H_2O production, at a given non-protein respiratory quotient, and the non-protein respiratory quotient is estimated from the food quotient (carbohydrate and fat content of food) or, alternatively, by respiratory gas exchange.

[0054] Steady state methods are generally more straightforward for calculation purposes than die-away curves, where multiple samples and fitting to an approximate isotope curve may be needed. The steady-state method is generally recommended over the die-away method for greater accuracy of measurement, although each method may have advantages in certain circumstances.

[0055] The methods of measuring energy expenditure described herein can be used to determine the energy expenditure at a timepoint. In an alternative format, the measurements can be taken over time or in replicates. Because the measurement are dependent on water turnover, other factors, such as nocturnal habits, access to drinking water, may be considered to the extent that it affects water turnover.

Measuring Whole-Body Caloric Balance

[0056] A measurement of whole-body calorie balance or, more specifically, whole body fat balance, can also be made, based on the dilution of exogenous (labeled) water by endogenous (unlabeled) water (this application can only be performed with hydrogen labeled water (*e.g.*, ^2H_2O or ^3H_2O)). In order to more fully understand the invention, the following brief background concerning whole-body calorie balance is provided: (1) If an individual (*e.g.*, animal or human) is in a state of persistent negative caloric balance (*i.e.*, for more than a few days), this generally reflects the loss of body fat (rather than body carbohydrates, for example), because fat represent the only non-protein nutrient store capable of providing more than a day or two of energy for the body. (2) Body fat has a higher caloric density (*i.e.*, more

calories per gram) than lean tissue, due to the more reduced chemical state and the lack of hydration of stored fat. Accordingly, concurrent loss of body fat with gain of lean tissue may occur under certain circumstances and result in no change in body weight, despite net loss of caloric content of the body (*i.e.*, negative caloric balance). Conversely, loss of lean tissue with gain of fat may occur and result in body weight loss, despite net retention of calories by the body (*i.e.*, positive calorie balance). (3) The hydrogen atoms in stored body fat do not generally exchange with body H_2O, so these hydrogen atoms in stored body fat remain almost entirely unlabeled when ^2H_2O or ^3H_2O water is administered, and the process of mobilizing and combusting body fat for fuel results in dilution of body ^2H_2O or ^3H_2O water. (4) The production of metabolic water, resulting from combustion of stored fuels in the body, can take the place of exogenous water (drinking water), in the body's total water needs. Ad-libitum (at pleasure) input of labeled (exogenous) water can therefore decrease quantitatively, when body fat is mobilized and oxidized, in individuals given ^2H_2O or ^3H_2O.

[0057] Reduction in body water enrichments during the ad-libitum intake of ^2H_2O or ^3H_2O therefore can be used as an index of body fat combustion and negative fat (and caloric) balance. This applies, even when body weight is stable (*i.e.*, if body composition is changing, with fat being oxidized and lean body mass being stored). A reduction in body water ^2H_2O or ^3H_2O enrichment during ad-libitum administration of ^2H_2O or ^3H_2O in drinking water, therefore represents an objective biomarker of negative fat and caloric balance (oxidation of body fat).

IV. Methods of Use

[0058] The invention described herein can be used for a variety of different uses. In one embodiment, the methods disclosed herein can be used for measuring total energy expenditure in the screening and testing of pharmacologic agents for thermogenic actions (*i.e.*, actions that increase the expenditure of energy, or the burning of calories) on cells or individuals. Pharmacologic agents include, but are not limited to, any chemical compound or composition disclosed in the 12th Edition of *The Merck Index* (a U.S. publication, Whitehouse Station, N.J., USA). For testing

either one or more cells (e.g. a cell culture) or testing in an individual, the energy expenditure is monitored before and after the administration of the pharmacologic agent to determine the effect or efficacy of the agent on the cells or individuals. As with any testing for effects or efficacy, a protocol that involves step-wise increment or decreases in the amount of agent administered is generally used. The methods described herein are useful for high throughput screening of pharmacologic agents.

[0059] In another embodiment, the methods disclosed herein can be used for measuring total energy expenditure in the screening and testing of genes that exert thermogenic actions in cells or individuals. In one format, genetically modified rodents are tested. In another format, human cells are tested. This is accomplished by measuring energy expenditure for a population of cells or individuals and correlating the effects of the expression (increased/decreased) of certain genes or combinations of genes on energy expenditure.

[0060] A number of methods have been used to obtain transgenic, non-human animals, which are animals that have gained an additional gene by the introduction of a transgene into their cells (e.g. both the somatic and germ line cells). In some cases, transgenic animals can be generated by commercial facilities (e.g. The Transgenic Drosophila Facility at Michigan State University, The Transgenic Zebrafish Core Facility at the Medical College of Georgia (Augusta, Georgia), and Xenogen Biosciences (St. Louis, MO)). In general, the construct containing the transgene is supplied to the facility for generating a transgenic animal.

[0061] Methods for generating transgenic animals are well known to those of skill in the art. Such methods include introducing the transgene into the germ line of the animal. For example, the transgene may be microinjected into the pronucleus of an early stage embryo. Alternatively, the transgene can be introduced into the pronucleus by retroviral infection. Other approaches include insertion of transgenes into viral vectors including recombinant retrovirus, adenovirus, adeno- associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly. Other approaches include delivering the transgenes, in the form of plasmid DNA, with the help of, for example, cationic

liposomes (lipofectin) or derivatized (e. g. antibody conjugated) polylysine conjugates, gramicidin S, artificial viral envelopes, or other such intracellular carriers, as well as direct injection of the transgene construct or $CaPO_4$ precipitation carried out in vivo. "Knock-out" mice can be analyzed using the techniques of the invention.

[0062] For a review of techniques that can be used to generate and assess transgenic animals, skilled artisans can consult Gordon, (M. Rev. Cytol. 115: 171-229, 1989), and may obtain additional guidance from, for example, Hogan et al. (Manipulating the Mouse Embryo," Cold Spring Harbor Press, Cold Spring Harbor, NY, 1986; Krimpenfort et al., Biotechnology 9: 86,1991 ; Palmiter et al., Cell 41: 343, 1985 ; Kraemer et al., "Genetic Manipulation of the Early Mammalian Embryo, "Cold Spring Harbor Press, Cold Spring Harbor, NY, 1985; Hammer et al., Nature 315: 680,1985 ; Purcel et al., Science 244: 1281, 1986; Wagner et al., U.S. Patent No. 5,175,385, and Krimpenfort et al, U.S. Patent No. 5,175,384). Such work in transgenic animals can be utilized to identify key genes involved in energy expenditure. The genes may also serve as targets for drug use in humans.

[0063] In another embodiment, the methods disclosed herein can be used for monitoring physiological conditions, such as diabetes or obesity. The monitoring of energy expenditure over time can give indications of decrease or increase in metabolic rate, which may be indicative of a physiological condition that has already developed.

[0064] In another embodiment, the methods disclosed herein can be used to monitor athletic progress, for example, in a weight training program. Alone or in combination with other indicators (*e.g.*, weight, body fat measurement, etc.), the increase or decrease of energy expenditure can provide an indication of increased metabolism, relative fitness gain, or of the total exercise activity in fact achieved by the program.

[0065] In yet another embodiment, the methods disclosed herein can be used to identify the presence of negative caloric balance in an individual. This can be accomplished by first determining metabolic water production and ^2H_2O enrichment

of body water in an individual prior to exposure to an intervention, then measuring metabolic water production and ^2H_2O enrichment of body water after the intervention, and then monitoring the ^2H_2O enrichment of body water relative to drinking water. The intervention can include, but is not limited to, administration of an agent, presence of one or more transgene(s), and participation in an exercise regimen or dietary regimen. A decline in ^2H_2O enrichment of body water relative to drinking water is indicative of fat mobilization and negative whole-body fat and caloric balance, even in the presence of stable or increasing body weight.

Kits

[0066] The invention provides kits for carrying out the methods of the invention. Accordingly, a variety of kits are provided in suitable packaging. The kits may be used for any one or more of the uses described herein, and, accordingly, may contain instructions for any one or more of the following uses: measuring energy expenditure in individuals and measuring energy expenditure in one or more cells (e.g. a cell culture).

[0067] The kits of the invention comprise one or more containers comprising any of the materials, for example, labeled water (*e.g.*, ^2H_2O, ^3H_2O, $H_2{}^{18}O$), described herein. Each component (if there is more than one component) can be packaged in separate containers or some components can be combined in one container where cross-reactivity and shelf life permit. Generally, labeled water is very stable and may be stored long term. The kit can optionally include dispensers for administering labeled water (*e.g.*, such that volume is accurately measured) and tools for obtaining a sample of biological fluids, cellular medium (e.g. cell culture medium).

[0068] The kits of the invention may optionally include a set of instructions, generally written instructions, although electronic storage media (*e.g.*, magnetic diskette or optical disk) containing instructions are also acceptable, relating to how to perform the measurement of metabolic water production and total energy expenditure. The instructions included with the kit generally include information as to the components and their administration to an individual. The kit may also be

commercialized as part of larger package that includes instrumentation for measuring isotopic contents of the labeled water, for example, a mass spectrometer.

[0069] The following is provided to illustrate but not to limit the invention.

EXAMPLES

Example 1 Animal Studies

[0070] Rats were administered 4% ^2H_2O as their drinking water (^2H_2O mixed with H_2O, by weight). After 1-12 weeks, blood or urine samples were collected (about <0.2 ml per sample). The isotopic enrichment or body water observed was about 2.8-3.0% (Figure 2), not 4%, showing a dilution of ingested water. Animals were on dry food diets, thus the moisture in food could not account for the dilution. It was very likely that metabolic water production accounted for the dilution relative to drinking water. As shown in Figure 2, the dilution by metabolic water production was constant over the course of several months.

Example 2 Animal Studies with an Intervention

[0071] Genetically obese mice (ob/ob, n=3), were administered 8% ^2H_2O as their drinking water (^2H_2O mixed with H_2O, by weight). Urine samples were collected daily and intake of drinking water was measured daily. After one week, a subcutaneous mini-osmotic pump containing recombinant leptin (Amgen, Inc.) was implanted surgically. Urine samples were then collected daily for 12 days and water intake was measured every day. Metabolic water production was calculated before and after administration of recombinant leptin (Figure 3A). An increase in metabolic water production was clearly evident, as was a decrease in ^2H_2O enrichment in body water in the group on average (Figure 3B) as well as each individual animal (Figure 3C). Recombinant leptin is known to increase total energy expenditure in *ob/ob* mice, consistent with these results for metabolic water production (Figure 3A), using the methods described herein. Moreover, recombinant leptin is known to cause loss of body fat with increase in lean body mass (*e.g.*, altering the composition of the body), thereby resulting in negative caloric balance, mobilization and oxidation of

body fat stores, and marked decrease in ^2H_2O enrichments in body water (Figures 3B and 3C).

Example 3 Human Studies

[0072] AIDS patients and healthy volunteers were given 50 ml of 70% ^2H_2O by mouth twice a day. Between days 5-60 of this regimen, blood, saliva, or urine were collected. Isotopic enrichment of body water was calculated and shown in Figure 4. The calculated value of total body water turnover from these individuals was 3.5-5.0 L/day. These values are considerably greater than the exogenous water intake values usually given in textbooks (~2.5L) (*e.g.*, Harrison's Internal Medicine, 2001). The difference between exogenous water intake and total body water turnover represents metabolic water production, as explained above. Metabolic water production in humans is therefore about 1L/day. Data from a large number of individuals are shown in Figure 5. Body water enrichment were generally <2.0 %, again consistent with body water flux >3.5 L, thus showing that metabolic water production was consistently measurable in humans.

[0073] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application. All publications, patents and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent or patent application were specifically and individually indicated to be so incorporated by reference.

[0074] All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, or patent application were specifically and individually indicated to be so incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and

modifications may be made thereto without departing from the spirit and scope of the appended claims.

Table 1 Caloric Values for Metabolic Water Production (ml H_20 produced) at Non-Protein Respiratory Quotient or Food Quotient

Nonprotien Respiratory Quotient or Food Quotient	Caloric Value of 1 ml of H_2O Produced	Percentage of Total Heat Produced by Fat
0.707	8.826	100.0
0.71	8.258	98.5
0.72	8.164	95.2
0.73	6.458	91.6
0.74	7.985	88.0
0.75	7.899	84.4
0.76	7.816	80.8
0.77	7.734	77.2
0.78	7.654	73.7
0.79	7.578	70.1
0.80	7.501	66.6
0.81	7.428	63.1
0.82	7.355	59.7
0.83	7.286	56.2
0.84	7.218	52.8
0.85	7.151	49.3
0.86	7.086	45.9
0.87	7.021	42.5
0.88	6.960	39.2
0.89	6.899	35.8
0.90	6.839	32.5
0.91	6.780	29.2
0.92	6.723	25.9
0.93	6.666	22.6
0.94	6.613	19.5
0.95	6.559	16.0
0.96	6.506	12.8
0.97	6.456	9.51
0.98	6.405	6.37
0.99	6.356	3.18
1.00	6.309	0

Modified from N. Zuntz and H. Schumberg, with modifications by G. Lusk, E.P. Catheart, and D.P. Cuthbertson, *J. Physiol. (London)* 72:349 (1931).

I claim:

1. A method of determining energy expenditure in an individual comprising the steps of:

(a) administering to the individual an amount of labeled exogenous water for a time sufficient for the label to reach a steady-state isotopic content in the individual;

(b) obtaining a biological sample from the individual;

(c) measuring the isotopic content of water in the biological sample;

(d) comparing the isotopic content of water in the biological sample to the isotopic content in the labeled exogenous water administered to the individual in step (a) to determine the total dilution rate of the label in total body water; and

(e) calculating the production rate of metabolic water from the total dilution rate to determine the energy expenditure in the individual.

2. The method of claim 1 wherein the labeled water is $^{2}H_{2}0$, $^{3}H_{2}0$, or $H_{2}O^{18}$.

3. The method of claim 2, wherein the labeled water is $^{2}H_{2}0$.

4. The method of claim 1 wherein the labeled water is administered orally.

5. The method of claim 1 wherein the biological sample is selected from the group consisting of urine, blood, saliva, interstitial fluid, edema fluid, lacrimal fluid, inflammatory exudates, synovial fluid, abscess, emphysema, cerebrospinal fluid, sweat, pulmonary secretions, seminal fluid, feces, bile, and intestinal secretions.

6. The method of claim 1 wherein the isotopic enrichment of in the labeled water is determined by isotope-ratio mass spectrometry, GC/MS, cycloidal/MS, or Fourier-Transform Infrared Spectroscopy (FTIR).

7. The method of claim 1 wherein the individual is a rat, mouse, other experimental animal, or human.

8. The method of claim 7 wherein the individual is human.

9. A method of determining energy expenditure in an individual comprising:

(a) administering labeled water to the individual;

(b) discontinuing said administering step (a);

(c) obtaining two or more biological samples from the individual wherein said biological samples comprise endogenous water and said endogenous water includes labeled water administered in step (a);

(d) measuring the isotopic content of the endogenous water in the biological samples;

(e) determining the decay rate of the labeled water in said biological samples to determine the total dilution rate of body water in the individual; and

(f) calculating the production rate of metabolic water from said total dilution rate to determine the energy expenditure in the individual.

10. The method of claim 9 wherein the labeled water is $^{2}H_2O$, $^{3}H_2O$, or H_2O^{18}.

11. The method of claim 10 wherein the labeled water is $^{2}H_2O$.

12. The method of claim 9 wherein the labeled water is administered orally.

13. The method of claim 9 wherein the biological sample is selected from the group consisting of urine, blood, saliva, interstitial fluid, edema fluid, lacrimal fluid, inflammatory exudates, synovial fluid, abscess, emphysema, cerebrospinal fluid, sweat, pulmonary secretions, seminal fluid, feces, bile, and intestinal secretions.

14. The method of claim 9 wherein the isotopic enrichment of in the labeled water is determined by isotope-ratio mass spectrometry, GC/MS, cycloidal/MS, or Fourier-Transform Infrared Spectroscopy (FTIR).

15. The method of claim 9 wherein the individual is an experimental animal or a human.

16. The method of claim 15 wherein the individual is a human.

17. A method of determining energy expenditure of one or more cells *in vitro* comprising the steps:

(a) incubating the one or more cells *in vitro* in a medium containing a known isotopic content of labeled water;

(b) obtaining a sample of said medium after a known period of time;

(c) measuring the isotopic content of water in said sample;

(d) calculating the rate of decline in the isotopic content of the labeled water in the cellular medium over the known period of time; and

(e) calculating the production rate of metabolic water by said one or more cells to determine the energy expenditure of said one or more cells.

18. The method of claim 17 wherein the labeled water is $^{2}H_20$, $^{3}H_20$, or H_2O^{18}.

19. The method of claim 18, wherein the labeled water is $^{2}H_20$.

20. The method of claim 17 wherein the biological sample is selected from the group consisting of urine, blood, saliva, interstitial fluid, edema fluid, lacrimal fluid, inflammatory exudates, synovial fluid, abscess, emphysema, cerebrospinal fluid, sweat, pulmonary secretions, seminal fluid, feces, bile, and intestinal secretions.

21. The method of claim 15 wherein the isotopic enrichment of ^{2}H or ^{18}O in the labeled water is determined by isotope-ratio mass spectrometry, GC/MS, cycloidal/MS, or Fourier-Transform Infrared Spectroscopy (FTIR).

22. The method of claim 17 wherein the cells are isolated from a rat, mouse, other experimental animal, or human.

23. The method of claim 22 wherein the cells are human cells.

24. A method for identifying a pharmacologic agent having a thermogenic action comprising the steps of:

(a) determining the energy expenditure in an individual according to claim 1 prior to exposure of the individual to the agent;

(b) exposing the individual to the pharmacologic agent;

(c) determining the energy expenditure in the individual according to claim 1 after exposure to the pharmacologic agent; and

(d) determining the energy expenditure in the individual before and after exposure to the agent, wherein an increase in energy expenditure after exposure to the pharmacologic agent identifies the pharmacologic agent as having a thermogenic action.

25. A method for identifying a pharmacologic agent having thermogenic action comprising the steps of:

(a) determining the energy expenditure in one or more cells according to claim 1 prior to exposure of said one or more cells to the pharmacologic agent;

(b) exposing the one or more cells to the pharmacologic agent;

(c) determining the energy expenditure in the one or more cells according to claim 1 after exposure to the pharmacologic agent; and

(d) determining the energy expenditure in the one or more cells before and after exposure to the agent, wherein an increase in energy expenditure after exposure to the pharmacologic agent identifies the pharmacologic agent as having a thermogenic action.

26. A method for identifying one or more genes involved in a thermogenic action in an individual comprising the steps of:

(a) determining the energy expenditure in the individual according to claim 1, wherein the individual has been genetically manipulated or an individual or is genetically well-characterized; and

(b) correlating the energy expenditure in the individual with the genetic composition or gene expression of the individual to thereby identify one or more genes involved in a thermogenic action in the individual.

27. A method for identifying one or more genes that are involved in thermogenic actions in one or more cells comprising the steps of:

(a) determining the energy expenditure in the one or more cells according to of claim 1, wherein the one or more cells are selected from the group consisting of a cell type, one or more cells that have been genetically manipulated, and one or more cells that are genetically well-characterized; and

(b) correlating the energy expenditure in the one or more cells with the genetic composition or gene expression of the one or more cells to thereby identify one or ore genes involved in thermogenic actions in the one or more cells.

28. The method of claim 26 wherein the individual has been genetically manipulated.

29. The method of claim 28, wherein the individual is a transgenic mouse or a knockout mouse.

30. The method of claim 28 wherein the individual that is genetically well-characterized is characterized by a gene expression chip or proteome measurements.

31. A method for identifying the presence of negative caloric balance in an individual comprising the steps of:

(a) determining metabolic water production and ^2H_2O enrichment of body water in an individual according to the method of claim 1 prior to exposure to an intervention;

(b) subjecting the individual to an intervention;

(c) measuring metabolic water production and ^2H_2O enrichment of body water according to the method of claim 1 after the intervention; and

(d) monitoring ^2H_2O enrichment of body water relative to drinking water, wherein a decline in ^2H_2O enrichment of body water relative to drinking water

is indicative of fat mobilization and identifies a negative whole-body fat and caloric balance.

32. The method of claim 31 wherein the intervention is administration of an agent, the presence of one or more transgene(s), or participation in an exercise regimen or dietary regimen.

33. A method of identifying or monitoring a disease or disorder, comprising:

a) determining the energy expenditure of the individual according to the method of claim 1 at a first timepoint; and

b) determining the energy expenditure of an individual according to the method of claim 1 at a second timepoint;

wherein a change the energy expenditure between said first timepoint and said second timepoint identifies the disease or disorder.

34. The method of claim 33, wherein the disease or disorder is diabetes mellitus or obesity or other disorder of energy balance.

35. A method of identifying a beneficial effect of an exercise regimen, comprising:

a) determining the energy expenditure of the individual according to the method of claim 1 at a first timepoint;

b) subjecting said individual to an exercise regimen; and

c) determining the energy expenditure of an individual according to the method of claim 1 at a second timepoint;

wherein a change in the energy expenditure between said first timepoint and said second timepoint identifies a beneficial effect of said exercise regimen.

36. A kit for determining energy expenditure in an individual or cells, comprising:

labeled water, and

instructions for using the kit,

wherein the kit is used to determine the energy expenditure of the individual or cells.

37. The kit of claim 36, further comprising instructions for performing energy expenditure calculations.

38. The kit of claim 36, further comprising tools for administering administering labeled water.

39. The kit of claim 36, further comprising tools for obtaining biological samples.

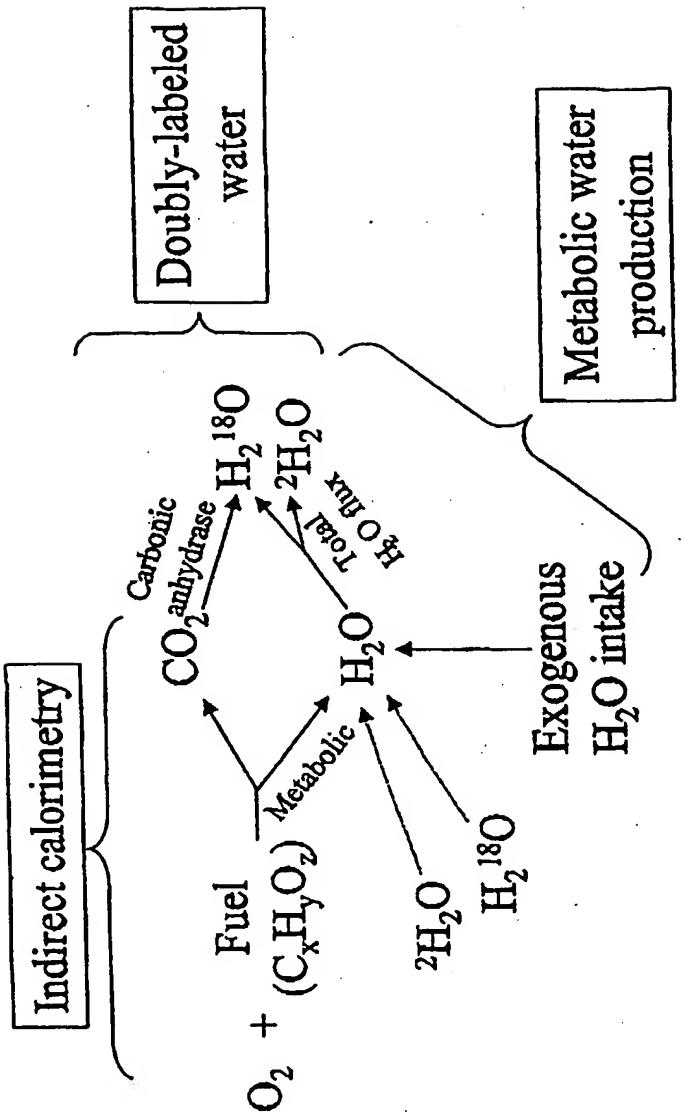
Indirect calorimetry
Doubly-labeled water
Metabolic water production
O_2 +
Fuel
$(C_xH_yO_z)$
Metabolic
Carbonic anhydrase
CO_2
$H_2{}^{18}O$
^2H_2O
Total H_2O flux
H_2O
^2H_2O
$H_2{}^{18}O$
Exogenous H_2O intake

FIGURE 1

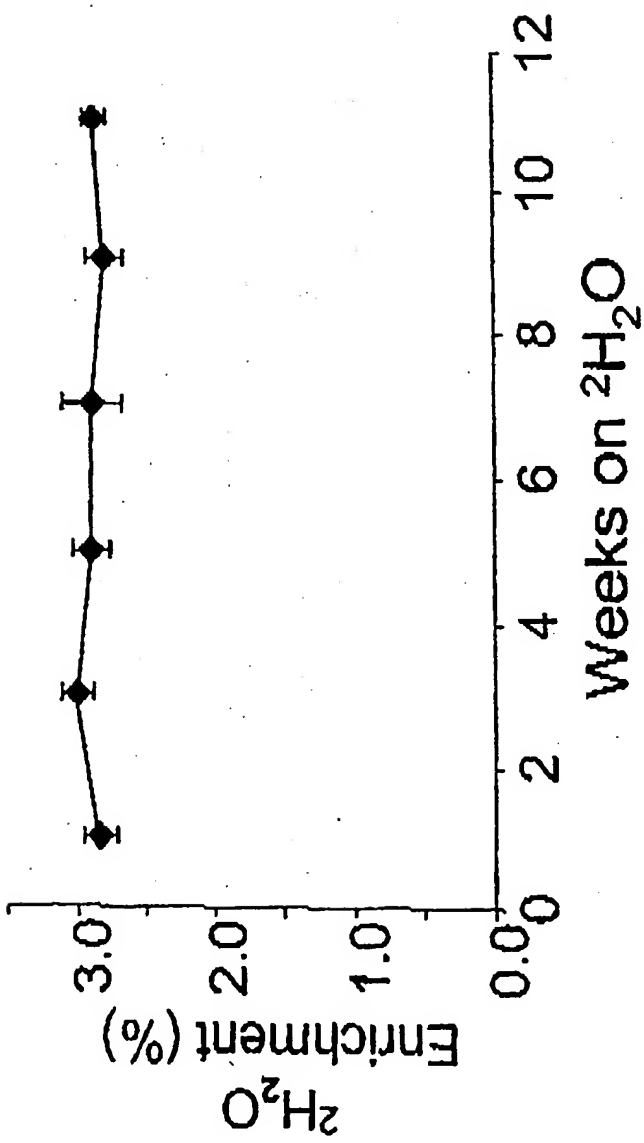

FIGURE 2

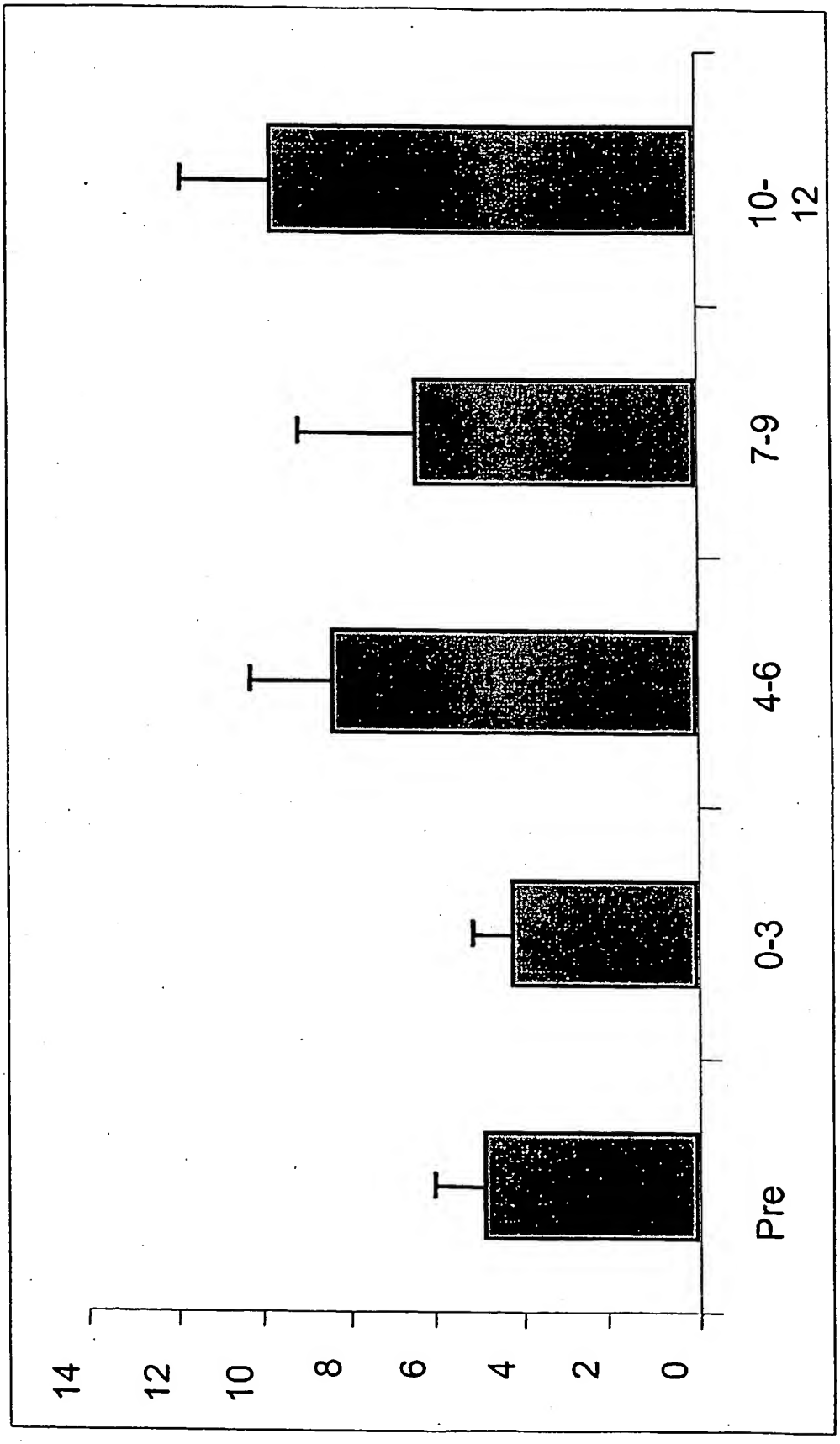

FIGURE 3A

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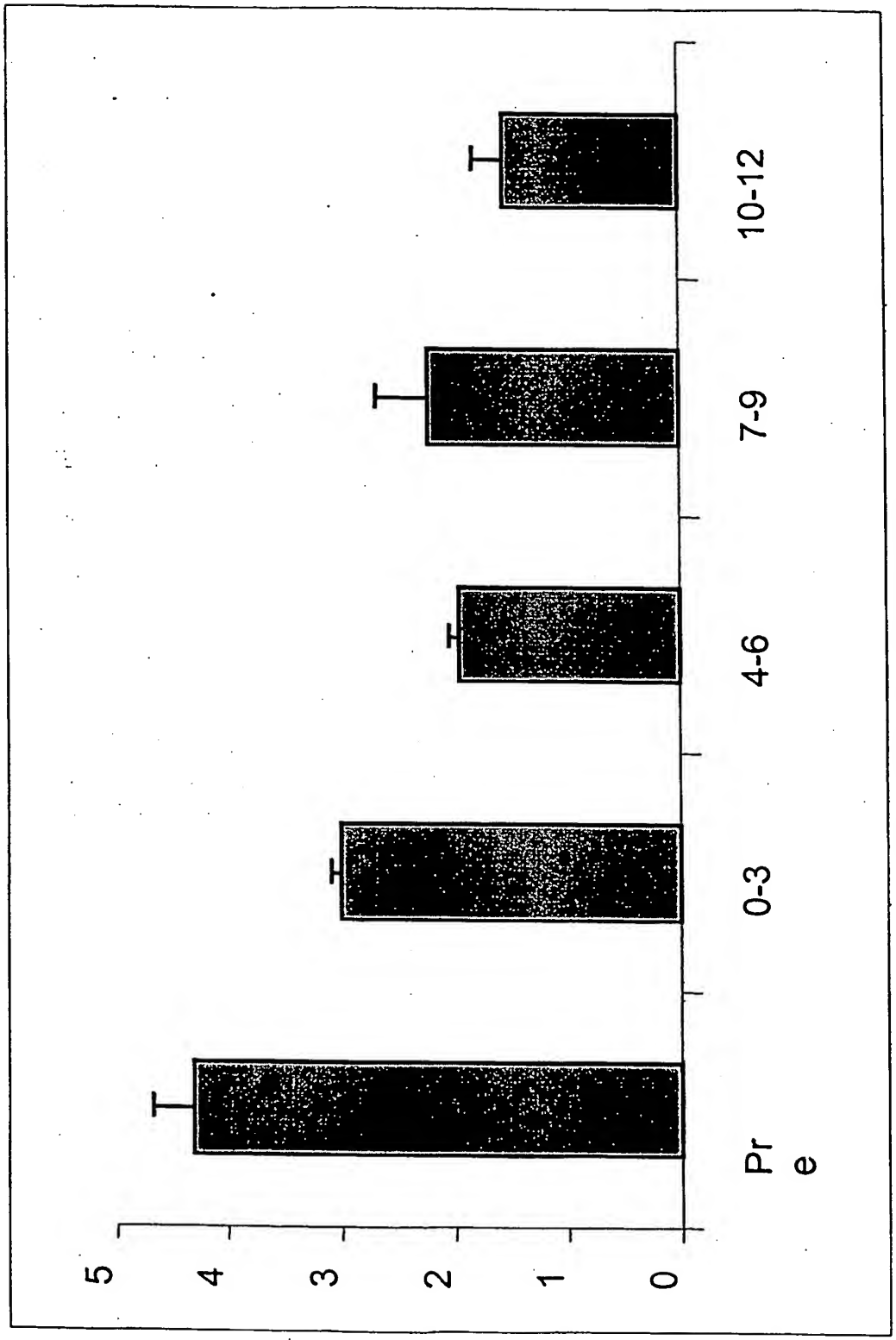

FIGURE 3B

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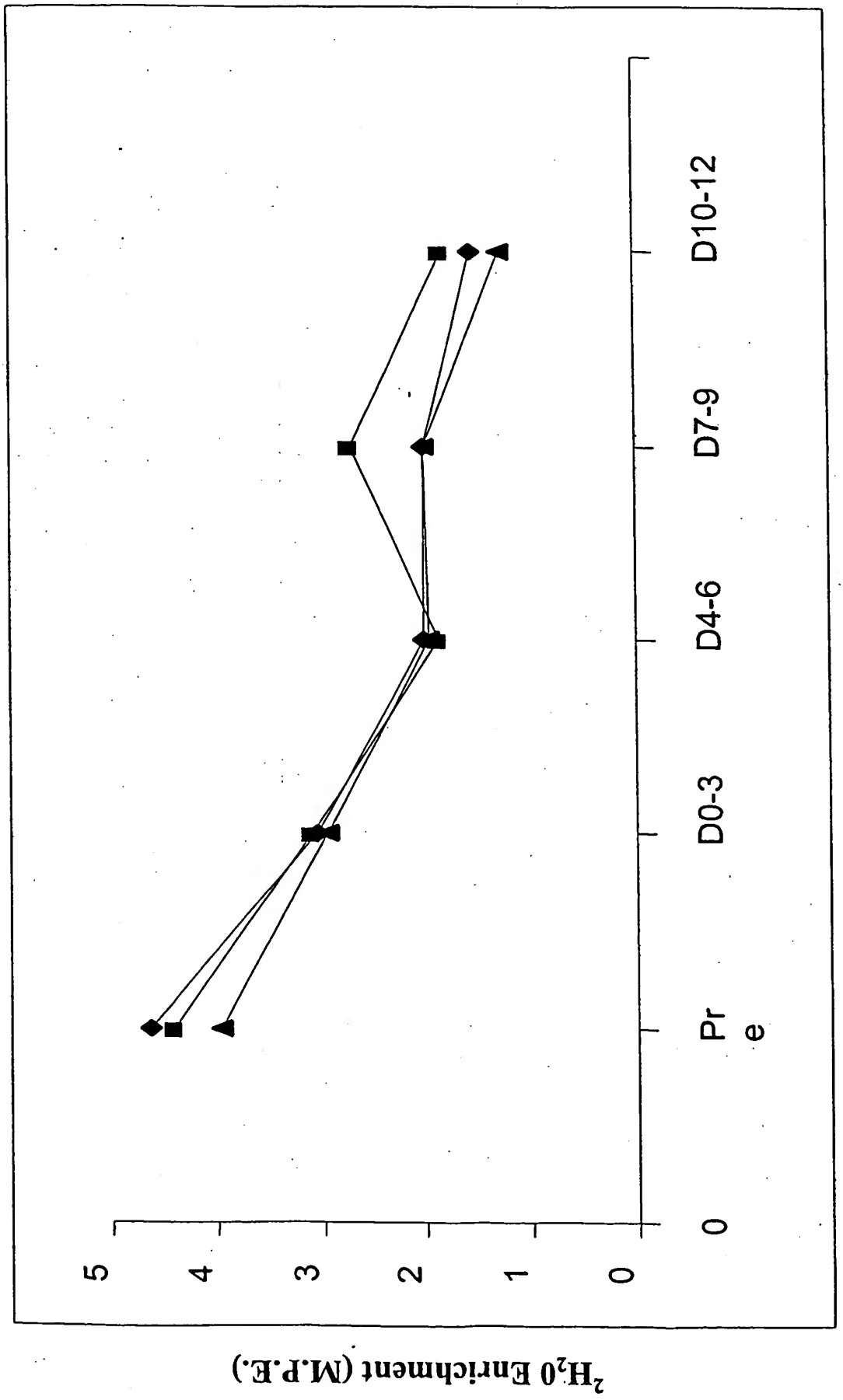
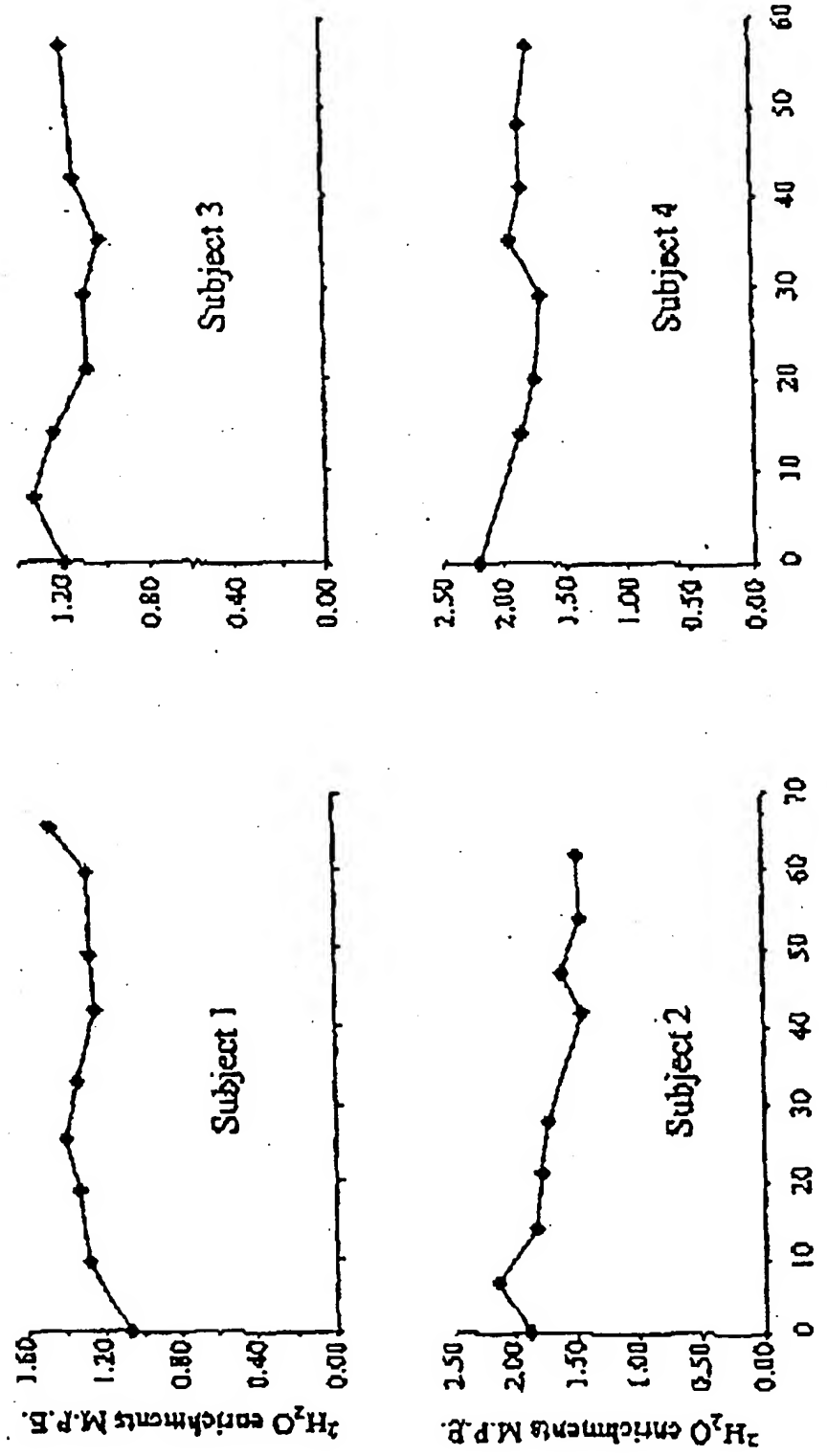

$^2H_20$ Enrichment (M.P.E.)
0
1
2
3
4
5
Pre
D0-3
D4-6
D7-9
D10-12
Day of Leptin Treatment

FIGURE 3C


Subject 1
Subject 2
Subject 3
Subject 4
^2H_2O enrichments M.P.B.
^2H_2O enrichments M.P.B.
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0.40
0.80
1.20
1.60
0.00
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2.00
2.50
0
10
20
30
40
50
60
70

FIGURE 4

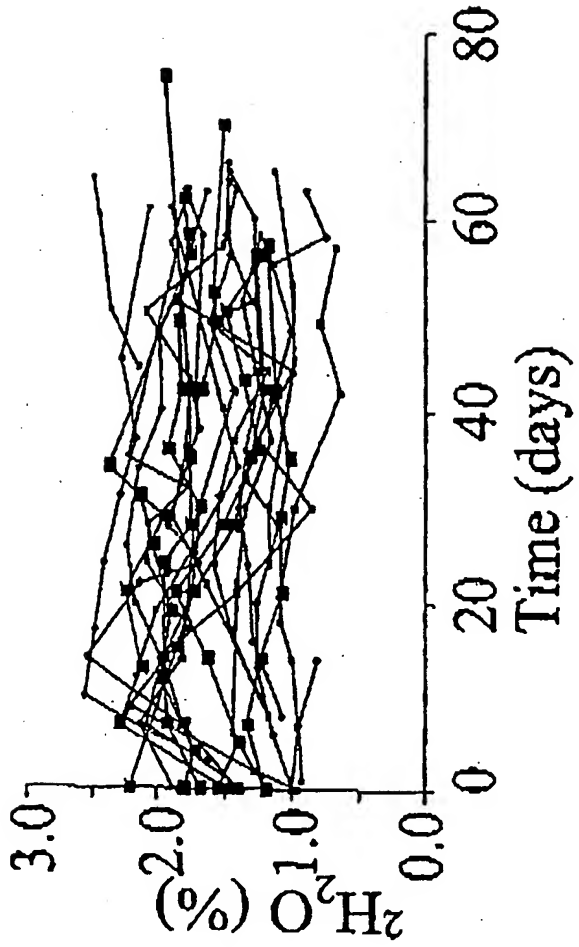

FIGURE 5

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